Pre-transplant thymic function is associated with the risk of cytomegalovirus disease after solid organ transplantation

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Abstract

Cytomegalovirus (CMV) disease is an important complication in solid organ transplant recipients. Thymic function in adults is associated with specific T-cell immunity. Pre-transplant thymic function was analysed in 75 solid organ transplant patients by the use of nested PCR. The primary outcome was the incidence of CMV disease 12 months after transplantation. Using multivariable logistic regression, we studied whether pre-transplant thymic function is an independent risk factor for CMV disease after transplantation. Thymic function was related to the risk of CMV disease in CMV-seropositive recipients. In these recipients, pre-transplant thymic function of <9.5 (OR 11.27, 95% CI 1.11–114.43, p 0.040) and the use of thymoglobulin (OR 8.21, 95% CI 1.09–61.84, p 0.041) were independent risk factors for CMV disease at 12 months after transplantation. Patients with pre-transplant thymic function values of <9.5 had a higher subsequent incidence of CMV disease (24%) than patients with values of ≥9.5 (3%) (log-rank test: 5.727; p 0.017). The positive and negative predictive values of these pre-transplant thymic function cut-offs were 0.24 (95% CI 0.10–0.45) and 0.97 (95% CI 0.82–1.00), respectively. Pre-transplant thymic function in CMV-seropositive candidates could be useful in determining the risk of post-transplant CMV disease in solid organ transplant patients, selecting a group of low-risk candidates.

Keywords: CMV-seronegative, CMV-seropositive, cytomegalovirus replication, solid organ transplantation, thymic function
Original Submission: 25 September 2014; **Revised Submission:** 20 December 2014; **Accepted:** 24 December 2014
Editor: G. Antonelli
Article published online: 14 January 2015

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Introduction

The risk of cytomegalovirus (CMV) disease and strategies for CMV prevention have traditionally been defined by the type of organ transplant, donor/recipient CMV serology, and the immunosuppression used [1–4]. Currently, the indication for preventive management is based on the recognition of these risk factors in the pre-transplant period. In recent years, remarkable advances have been made in understanding the risk factors, in the design of prevention strategies, and in new techniques for virological and immunological monitoring. The pre-transplant serological status of candidates is used as a surrogate marker for the presence of CMV-specific immunity [5,6]. However, there is evidence that serology is not the best method for defining the immune status of transplant candidates. In a previous study, we showed that the pre-transplant production of interferon (IFN)-γ

http://dx.doi.org/10.1016/j.cmi.2014.12.020

by CMV-specific T-lymphocytes is associated with the risk of CMV replication after transplantation. In that study, one-third of the CMV-seropositive recipients (R+) did not produce enough IFN-γ, and this was associated with an increased risk of CMV replication [7]. Identifying the immune characteristics of each individual therefore provides an opportunity to individualize preventive management [5,8].

Determining the protective capacity of the transplant recipient's immune system would also be of crucial importance.The thymus is critical for the formation of the immune system and T-cells during the fetal stage [9]. For years, it has been assumed that the generation of T-lymphocytes is fixed in childhood. However, some studies have shown that the adult thymus may reverse the process of atrophy to facilitate improved recovery of the immune system [10,11], and adult thymic tissue even maintains the thymopoietic capacity [12]. There is evidence of a relationship between thymic function and specific T-cell immunity in adults. CMV-specific T-cell immunity can predict the risk of CMV infection or disease [12–15]. We hypothesized that insufficient thymic function in solid organ transplant (SOT) candidates may increase the risk of CMV disease after transplantation. Therefore, a higher level of pre-transplant thymic function could mean greater control of virus replication in all patients. Also, a failure of thymic function during the pre-transplant period could be considered to be a major risk factor for the development of CMV replication and disease in the post-transplant period.

Materials and methods

Study design and population

A longitudinal study of a cohort of SOT patients from two centres of the REIPI network was carried out. Patients were eligible if they were aged >14 years. The CMV prevention protocol was as follows. Universal prophylaxis was used in heart (3 months), lung (6 months) and pancreas–kidney (6 months) transplant recipients, as well as in kidney and liver transplant recipients (3 months) considered to be at high risk (CMV-seropositive donor/CMV-seronegative recipient (D+/R−), and use of induction therapy with thymoglobulin). In the remaining patients, pre-emptive therapy was used. Monitoring of viral load (COBAS AmpliPrep/COBAS Amplicor; Roche Diagnostics, Branchburg, NJ, USA) was required at least weekly during hospitalization, twice monthly during the first 3 months, and monthly during the first year. Treatment was indicated for a minimum of 2 weeks when the viral load was >1500 copies/mL. The drugs used for both prophylaxis and pre-emptive therapy were intravenous ganciclovir/valganciclovir at recommended doses in each case, and doses were adjusted for renal function if needed. Immunosuppression was indicated according to the protocols of each centre.

The primary study endpoint was the incidence of CMV disease within 12 months after transplantation. We assessed the value of pre-transplant thymic function for predicting the risk of CMV disease. For the diagnosis of disease, we used the definitions established by the American Society of Transplantation for use in clinical trials [16]. In brief, CMV disease was defined as evidence of CMV infection with compatible symptoms. CMV disease was classified as tissue-invasive disease if there was evidence of localized CMV infection in a biopsy or another appropriate specimen, or as CMV syndrome if there was no such evidence.

The study was approved by the ethics committee of the aforementioned institution.

Thymic function analysis

Thymic function was determined by quantitative nested PCR in frozen blood samples from the pre-transplant study. Details of this technique have been published elsewhere [17]. Briefly, the samples are amplified in triplicate in a first conventional PCR. Two primary reactions are performed for each sample. In a PCR tube, the T-cell receptor excision circle (sj-TREC) is amplified (δTREC) with a pair of specific oligonucleotides. In the other PCR tube, six of the 13 possible βTRECs are amplified (six corresponding to cluster 1) in a multiplex reaction with six sense oligonucleotides that hybridize to specific areas on each TREC, and with an antisense oligonucleotide that hybridizes in the common area shared by six TRECs. The six sense oligonucleotides also have a random sequence on the 3′ end (T3 tail), which is needed to standardize the results. Each triplicate is amplified on a LightCycler 480 II with specific FRET probes as a detection method. A mixture of the first two PCRs (δTREC and six βTRECs) is diluted 1 : 10 and used for quantitative PCR. For amplification of βTREC, an antisense oligonucleotide that hybridizes to the common area shared by the six TRECs and a sense oligonucleotide that hybridizes to the T3 tail added to the six amplicons are used. Thus, an integrated signal of the six βTRECs is obtained with a single pair of oligonucleotides. The δTREC/βTREC ratio can be determined in a single reaction vial by the use of FRET probes with different wavelengths (Red-610 and Red-640).

Variables associated with CMV disease

We studied the potential relationship between the development of CMV disease at 12 months after transplantation and the following variables: age, gender, type of organ transplant, donor/recipient serology, prevention strategy, immunosuppression, and pre-transplant thymic function.

Statistical analysis

The proportions were compared by use of the chi-square statistic for contingency tables and Fisher's exact test when indicated. Quantitative variables were expressed as mean ± standard deviation or median and minimum–maximum values. Normality was checked with Shapiro–Wilk test. Mean and median values were compared by use of Student's t-test or the Mann–Whitney's U-test. Pearson correlation coefficients were also calculated. To study the risk factors for CMV disease, a bivariate analysis was performed with simple logistic regression followed by multivariate analysis with logistic regression. After methodical selection of the variables included in the multivariate model, variables found to be significant in the bivariate analysis and others considered to be clinically important were included in the multiple logistic regression model. The cut-offs of pre-transplant thymic function associated with the best sensitivity and specificity were analysed by means of receiver operating characteristic curve analysis. Given that values of <9.5 were associated with the risk of mortality in a previous study, we started from the assumption that this value could be a valid cut-off point to categorize thymic function [18]. The incidence of CMV disease according to the categorized pre-transplant thymic function was calculated by the use of Kaplan–Meier curves. Differences were assessed with the log-rank test. The performance of pre-transplant thymic function in detecting the risk of CMV disease was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). All analyses were performed with PASW Statistics 18 software (IBM Corporation, Armonk, NY, USA). p-Values were considered to be statistically significant at $p < 0.05$, and hypothesis tests were two-sided.

Results

Baseline characteristics of the patients included in the study

A total of 75 patients were enrolled in the study. The median age was 47 years (range 15–74 years). Sixty-one patients (81%) were male. Forty-seven patients (63%) received a kidney transplant, six patients (8%) a heart transplant, 11 patients (15%) a lung transplant, and 11 patients (15%) a pancreas–kidney transplant. The majority were R+ (57 patients, 76%), and 14 patients (19%) were D+/R–. Forty-four patients (59%) received universal prophylaxis, and 31 patients (41%) received pre-emptive therapy. Regarding baseline immunosuppression, 83% (62 patients) received tacrolimus and 17% (11 patients) cyclosporine. Nine patients (12%) received induction with thymoglobulin, and five (7%) with basiliximab. Six patients (8%) received mammalian target of rapamycin inhibitors. Ten patients (13%) received steroid boluses, owing to rejection.

Thymic function

The median pre-transplant thymic function was 13.1 (range 0.004–665.52). An inverse correlation between thymic function and age was observed ($r = -0.260$, p 0.025). The median thymic function was lower in candidates aged >50 years (7.8, range 0.2–117.8) than in candidates aged 15–50 years (17.3, range 0.004–665.52), with p 0.013. No differences were found in pre-transplant thymic function associated with CMV replication. A moderate positive correlation was observed between pre-transplant thymic function and day of onset of CMV replication ($r = 0.441$, p 0.017). This correlation was stronger in younger patients ($r = 0.680$, p 0.007). No correlation was observed between thymic function and peak viral load in patients with CMV replication.

Thymic function was lower in seronegative recipients than in seropositive patients (27.78 ± 27.57 vs. 42.48 ± 120.84, p 0.398). We also found no differences in pre-transplant thymic function of R+ patients associated with CMV replication. In R+ patients, there was a positive correlation between pre-transplant thymic function and the day of first detection of CMV replication ($r = 0.527$, p 0.017).

According to the receiver operating characteristic curve analysis, thymic function values of <9.5 have 86% sensitivity and 62% specificity for predicting subsequent CMV disease.

Risk factors for CMV disease

During the first year after transplantation, 29 patients had CMV replication (16 patients received universal prophylaxis and 13 patients pre-emptive therapy) at a median of 98 days (range 28–344 days). Twelve of 29 patients had CMV disease (seven viral syndrome, four colitis, and one pneumonitis) at a median of 129.5 days (range 28–285 days). Eight of 12 patients received universal prophylaxis and four patients received pre-emptive therapy. No episode of CMV disease was observed while patients were on prophylaxis.

Table 1 shows the variables studied as potential risk factors for CMV disease in the 75 patients analysed. In the final multivariate model, variables independently associated with the risk of CMV disease were serology D+/R– (OR 6.67, 95% CI 1.49–29.73, $p < 0.013$) and induction of immunosuppression with thymoglobulin (OR 6.00, 95% CI 1.07–33.53, $p < 0.041$) (Table 2). Although pre-transplant thymic function levels were significantly lower in patients who developed CMV disease (12.01 ± 12.43 vs. 44.08 ± 115.11, p 0.035), neither pre-transplant thymic function as a continuous variable or pre-transplant thymic function as an sj-TREC/βTREC ratio of <9.5

TABLE 1. Bivariate analysis of risk factors for cytomegalovirus disease in solid organ transplant recipients (*n* = 75)

	Cytomegalovirus disease			
Variable	**Disease (*n* = 12)**	**No disease (*n* = 63)**	**OR (95% CI)**	**p**
Age (years), mean ± SD	49 ± 17	46 ± 13	1.01 (0.97–1.06)	0.550
Age >50 years (*n* = 34)	6 (50)	28 (44)	1.25 (0.36–4.30)	0.723
Male gender (*n* = 61)	10 (83)	51 (81)	1.17 (0.23–6.08)	0.846
Serology D+/R– (*n* = 14)	5 (42)	9 (14)	4.29 (1.11–16.49)	0.034
Non-renal transplant (*n* = 30)	4 (33)	26 (41)	0.71 (0.19–2.61)	0.608
Prevention with pre-emptive therapy (*n* = 31)	4 (33)	27 (43)	0.67 (0.18–2.45)	0.541
Use of thymoglobulin (*n* = 9)	3 (25)	6 (9)	3.17 (0.67–14.98)	0.146
Use of basiliximab (*n* = 5)	2 (17)	3 (5)	2.00 (0.77–5.20)	0.155
Use of tacrolimus (*n* = 60)	10 (83)	53 (84)	1.00 (0.19–5.28)	0.999
Use of mycophenolate (*n* = 73)	11 (92)	62 (98)	0.18 (0.01–3.05)	0.234
Use of mTOR inhibitors (*n* = 6)	1 (8)	5 (8)	1.05 (0.11–9.92)	0.963
Use of bolus steroids (*n* = 10)	2 (17)	8 (13)	1.37 (0.25–7.45)	0.712
Thymic function <9.5 (*n* = 30)	7 (58)	23 (36)	2.44 (0.69–8.56)	0.165

Data are expressed as no. (%), unless stated otherwise.
mTOR, mammalian target of rapamycin; SD, standard deviation.

TABLE 2. Multivariate analysis of risk factors for cytomegalovirus disease in solid organ transplant recipients (*n* = 75)

	Initial model			Final model		
Risk factor	**Coefficient**	**Adjusted OR (95% CI)**	**p**	**Coefficient**	**Adjusted OR (95% CI)**	**p**
Age (per year)	0.02	1.02 (0.97–1.07)	0.500	—	—	—
Non-renal transplant	–0.35	0.71 (0.09–5.35)	0.735	—	—	—
Serology D+/R–	2.82	16.84 (2.33–121.56)	0.005	1.90	6.67 (1.49–29.73)	0.013
Prevention with pre-emptive therapy	0.71	2.04 (0.29–14.44)	0.477	—	—	—
Use of thymoglobulin	2.11	8.29 (0.79–86.63)	0.077	1.79	6.00 (1.07–33.53)	0.041
Thymic function <9.5	1.39	4.03 (0.81–20.01)	0.088	—	—	—

behaved as a risk factor for CMV disease in the final multivariate model (Table 2).

During the first year after transplantation, 20 (35.1%) R+ patients had CMV replication and seven (12.3%) had CMV disease at a median of 95.5 days (range 28–344 days) and 127 days (range 28–285 days), respectively. Table 3 shows the bivariate analysis of the variables studied as potential risk factors for CMV disease in the 57R + patients. Six of seven (86%) of the R+ patients with CMV disease had a pre-transplant thymic function of <9.5 (vs. 38% in those without disease, p 0.041). In R+ patients with induction with thymoglobulin, CMV disease was also more frequent (43% vs. 10%, p 0.033). In the final multivariate model (Table 4), pre-transplant thymic function of <9.5 (OR 11.27, 95% CI 1.11–114.43, p 0.040) and the use of thymoglobulin (OR 8.21, 95% CI 1.09–61.84, p 0.041) were independent risk factors for CMV disease. The area under the curve of this model was 0.82 (0.68–0.95).

Predictive value of thymic function

When the incidence of CMV disease was analysed according to the results of thymic function in R+ patients, those patients with

TABLE 3. Bivariate analysis of risk factors for cytomegalovirus disease in seropositive solid organ recipients (*n* = 57)

	Cytomegalovirus disease			
Variable	**Disease (*n* = 7)**	**No disease (*n* = 50)**	**OR (95% CI)**	**p**
Age (years), mean ± SD	53 ± 16	49 ± 11	1.03 (0.96–1.10)	0.460
Age >50 years (*n* = 30)	4 (57)	26 (52)	1.23 (0.25–6.07)	0.799
Male gender (*n* = 47)	5 (71)	42 (84)	0.48 (0.08–2.90)	0.421
Non-renal transplant (*n* = 20)	3 (43)	17 (34)	1.46 (0.29–7.26)	0.647
Prevention with pre-emptive therapy (*n* = 29)	3 (43)	26 (52)	0.69 (0.14–3.42)	0.652
Use of thymoglobulin (*n* = 8)	3 (43)	5 (10)	6.75 (1.16–39.20)	0.033
Use of basiliximab (*n* = 4)	1 (14)	3 (6)	1.62 (0.48–5.41)	0.436
Use of tacrolimus (*n* = 47)	6 (86)	41 (85)	1.02 (0.11–9.85)	0.983
Use of mycophenolate (*n* = 55)	6 (86)	49 (98)	0.12 (0.01–2.22)	0.156
Use of mTOR inhibitors (*n* = 5)	1 (14)	4 (8)	1.92 (0.18–20.11)	0.588
Use of bolus steroids (*n* = 8)	2 (29)	6 (12)	2.93 (0.46–18.63)	0.254
Thymic function <9.5 (*n* = 25)	6 (86)	19 (38)	9.79 (1.09–87.70)	0.041

Data are expressed as no. (%), unless stated otherwise.
mTOR, mammalian target of rapamycin; SD, standard deviation.

TABLE 4. Multivariate analysis of risk factors for cytomegalovirus disease in seropositive solid organ recipients ($n = 57$)

	Initial model			Final model		
Variable	**Coefficient**	**Adjusted OR (95% CI)**	**p**	**Coefficient**	**Adjusted OR (95% CI)**	**p**
Age (per year)	0.03	1.03 (0.92–1.15)	0.610	—	—	—
Non-renal transplant	3.17	23.69 (0.85–664.12)	0.063	—	—	—
Prevention with pre-emptive therapy	2.71	14.98 (0.81–276.40)	0.069	—	—	—
Use of thymoglobulin	5.09	162.07 (3.89–6748.40)	0.007	2.11	8.21 (1.09–61.84)	0.041
Thymic function <9.5	2.87	17.58 (0.93–333.77)	0.056	2.42	11.27 (1.11–114.43)	0.040

levels of <9.5 had a higher subsequent incidence of CMV disease (24%) than patients with levels of ≥9.5 (3%) (log-rank test: 5.727; p 0.017; Fig. 1.). The performance of the thymic function assay with the cut-off of 9.5 for predicting CMV disease was as follows: sensitivity 0.86 (95% CI 0.42–0.99), specificity 0.62 (95% CI 0.47–0.75), PPV 0.24 (95% CI 0.10–0.45), and NPV 0.97 (95% CI 0.82–1.00).

Discussion

The main conclusion of this study is that thymic function failure, defined as a pre-transplant sj-TREC/βTREC ratio of <9.5 (thymic function deficit), is a risk factor for CMV disease in CMV-seropositive SOT recipients. It is well known that innate and adaptive immunity play an important role in the control of CMV

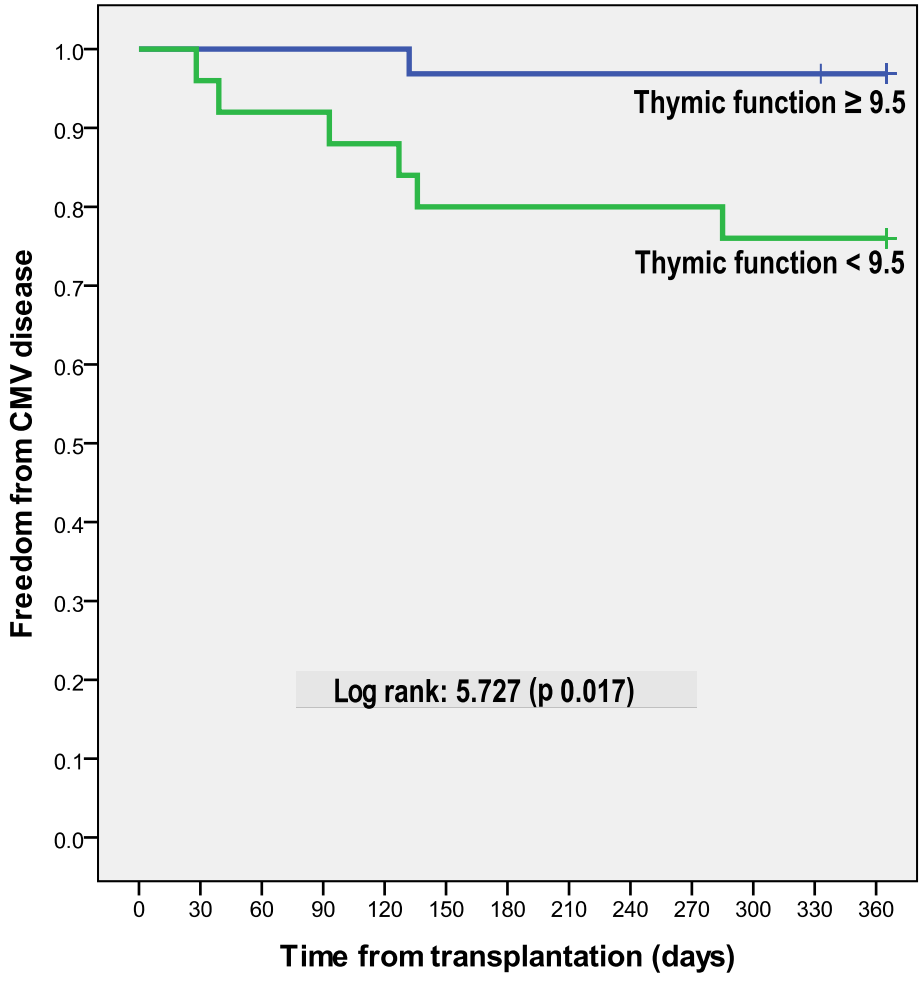

FIG. 1. Kaplan–Meier curves of the incidence of cytomegalovirus (CMV) disease according to the results of the pretransplant thymic function assay. Thymic function values: <9.5 vs. ≥9.5 (log-rank test: 5.727; p 0.017).

[19,20]. Some factors, such as Toll-like receptor (TLR)2 and TLR4 polymorphisms, have been associated with the risk of CMV disease [21]. The individual characteristics of the natural killer response also seem to be important [22–24]. Adaptive immunity plays an essential role, so that hypogammaglobulinaemia is a risk factor in heart and lung transplantation [25,26]. T-cell responses, particularly those of $CD4^+$ and $CD8^+$ T-cells, are critically important components of CMV immune control. Immune monitoring of CMV-specific T-cell responses can predict individuals at increased risk for CMV disease post-transplant, and may be useful in guiding prophylaxis and pre-emptive therapies. The majority of assays rely on the detection of IFN-γ after stimulation of whole blood or peripheral blood mononuclear cells with CMV-specific antigens or peptides. In addition to IFN-γ, other markers, including interleukin-2, tumour necrosis factor-α, CD107, and CD154, have been used to correlate CMV-specific T-cell responses with the risk of CMV.

There is evidence that a candidate's pre-transplant immunological status may be useful for defining the risk of CMV after transplantation. Although this has been classically done by identifying serological status, today we can determine the TLR polymorphisms [21] or the functionality of the CMV-specific T-cell response by measuring the production of INF-γ [7].

In the field of ageing, thymic function in adults and the elderly plays an active role in the maintenance of the peripheral virgin lymphocyte subpopulation [13]. Moreover, failure in the adult thymus is associated not only with a smaller proportion of naive lymphocytes, but also with the accumulation of cellular defects that potentially decrease lymphocyte function [15]. Also, levels of thymic function are independently associated with crude mortality in healthy elderly individuals [18]. In our study, we found that the lack of thymic function in CMV-seropositive candidates is associated with an increased risk of CMV disease. Our observation possibly reflects the importance of thymic function in adults to maintain CMV-specific immunity in individuals in whom it is assumed to have memory. It seems reasonable to assume that patients with poor thymic function become high-risk patients, as we found that six of the seven seropositive recipients with CMV disease had a thymic function of <9.5. This value has been associated with increased mortality in the healthy elderly [18], and suggests that 'thymic failure' may be indicative of an impaired immune system that has deteriorated too much to control CMV replication.

Another risk factor for the development of CMV disease is the type of prevention therapy (patients receiving pre-emptive therapy as compared with universal prophylaxis). Different studies [6,8] have established that both strategies are effective in preventing CMV disease during the time in which they are carried out (3–6 months). In D+/R− patients, universal prophylaxis favours the development of late disease after discontinuation of prophylaxis. Obviously, the strategies are not equal in terms of asymptomatic replication. Pre-emptive therapy does not prevent asymptomatic replication, as it is the marker used to start treatment. Therefore, the strategies are not equivalent in terms of avoiding asymptomatic CMV replication, and that is why we have not performed the analysis in terms of CMV replication but in terms of CMV disease. In our opinion, the posibility that prevention strategy is acting as a confusing variable is controlled by including it in the multivariate analysis. Thus, independent variables included in the multivariate analysis are controlled by the type of prevention.

In a previous study, we found that one-third of CMV-seropositive candidates have deficient CMV-specific T-cell function, as measured by the production of INF-γ, and this deficit is associated with the increased risk of replication after transplantation [7]. It is necessary to study whether the functional deficit in CMV-specific T-cell immunity could be related to impaired thymic function in SOT candidates. In this study, however, thymic function was not associated with the risk of CMV replication. A correlation was not found between the intensity of replication and pre-transplant thymic function. This may be because 59% of the entire cohort and 49% of CMV-seropositive recipients were given universal prophylaxis, thereby inhibiting viral replication. However, lower pre-transplant thymic function was found to be associated with earlier onset of CMV replication.

Owing to its high NPV, inclusion of the study of thymic function in assessing pre-transplant CMV-seropositive candidates could aid in identifying a subgroup of low-risk patients suitable for individualized CMV prevention. Nevertheless, it is premature to base any recommendation for changing CMV prevention strategies on the basis of our data. The thymic function value seems to be a supplementary tool for the prediction of patients at high risk of developing CMV disease. However, the PPV is very low as reported (0.24), and it is difficult to validate the use of 9.5 as a universal cut-off in R + SOT recipients. Additional interventional studies based on this and other markers of risk are necessary to determine whether patients could benefit from not performing prevention in low-risk organs or of performing pre-emptive therapy rather than universal prophylaxis in high-risk organs.

Transparency declaration

The authors declare that they have no conflicts of interest.

Author contributions

I. Gracia-Ahufinger, S. Ferrando-Martínez, S;. Cantisán, and M. del Carmen Muñoz-Villanueva: data collection and analysis, and

writing and final approval of the manuscript. M Montejo, A. Rivero, and R. Solana: patient care, data analysis, and writing and final approval of the manuscript. M. Leal and J. Torre-Cisneros: patient care, conception and design of the study, data collection and analysis, financial support, and writing and final approval of the manuscript.

Acknowledgements

This study was supported by Plan Nacional de I+D+i 2008-2011 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015), co-financed by the European Development Regional Fund 'A way to achieve Europe' ERDF, Redes Temáticas de Investigación en SIDA (ISCIII RETIC RD12/0017/0029 and RD12/0017/0037), Proyecto de Excelencia (CTS-6313), and Consejería de Salud (PI-0278). S. Ferrando-Martínez received a grant from the Fondo de Investigaciones Sanitarias (CD10/00382). This work was presented as an oral communication (#372) at the XVIII Meeting of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), May 2014, Valencia, Spain.

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